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Enrichment of Omega-3 Fatty Acids using Urea Complexation Method to Enhance the Nutritive Value of Stingray Fish (*Dasyatis Sephen* F.) Liver Oil

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ABSTRACT

Lipid fraction extracted from tissues of oily fish and fishery by-products are one of the best source of omega-3 (ω-3) polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Method of oil extraction from the Dasyatis sephen liver is simple and cheap. Therefore, the present study was conducted to extract and enrich the Omega-3 fatty acids from Dasyatis sephen liver which is discarded during dry fish production. Bligh and Dyer's method was used to extract oil. Fatty acids profiles were determined by Gas-Liquid Chromatography method (GLC). Average liver lipid recovery was 69.54 % (w/w). Crude liver oil fatty acid profile of DHA and EPA was 0.5% and 0.6%, respectively. Urea complexation was done to enrich the extracted Omega-3 fatty acids. Physio-chemical properties such as moisture content, color, specific gravity, peroxide value, and fatty acid compositions were obtained under the tolerable standard. The level of DHA:22:6n-3 and EPA: 20:5n-3 in the enriched Dasyatis sephen oil were 9.7 % and 8.7 %, respectively. It could be concluded that the converting of Ray fish by-product into enriched oil is an opportunity of adding value to the fish by-product and could be suitable for applications in pharmaceutical and nutraceutical industries.

KEYWORDS: *Fatty acid, Fish Oil, Omega-3, Ray Fish, Urea Complexation*

1. INTRODUCTION

Fish oil is the lipid fraction extracted from tissues of oily fish and fishery by-products and it is the best source of omega-3 (ω-3) polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

This paper describes the study performed on Ray fish liver oils from fish by-products discarded from a dry fish processing industry where usually discarded.

Even though refined fish oil has a numerous health benefits, only certain people afford to consume it as fish oil is an expensive item. Presently, the fish production is becoming more demanding as there is a sizable and growing world market demand for high quality fish oil. Apart from its various uses as consumable oil, it is also appreciable in both pharmaceuticals and food industries. Past literature has shown that little work has been done in terms of Ray fish liver oil and its fish by-products. Therefore, Ray fish liver oil may have applications in food, feed manufacturing and other industries via improve nutritive value of crude ray fish live oil.

2. Materials and Methods

2.1 Study Location

This study was conducted in the Fisheries Harbors of Gurunagar and Delft Islands, Jaffna, Northern Provenience in Sri Lanka. Analysis was conducted in the Nutrition Laboratory, Meat Science Laboratory, Department of Animal Science, Food Science Laboratory, Department of Food Science, Faculty of Agriculture, and University of Peradeniya and in the Beuro Veritas, Colombo, Sri Lanka.

2.2 Sample Collection and Preparation

Samples of Three Stingray fish *Dasyatis sephen* (Forsskal, 1175) were collected from each Fisheries Harbors and Dry Fish Cottages of Jaffna. They were transported in sealed freezer containers to laboratory at (-8 ˚C) and stored at (-20 ˚C) in the deep freezer for analyses. Each were taken out to thaw at room temperature to measure its lengths and weights as whole. Samples were eviscerated and its livers, offal (gut & tail) and carcass were stored at (-20 °C) in sealed in poly bags separately for further analysis.

Plate 2.1: Sting Ray (*Dasyatis sephen*)(Dorsal View)

2.2.2 Extraction of Ray Fish Liver Crude Oil

A modified Bligh and Dyer method (1959) was used to determine the crude lipids in the liver samples of ray fish. Extraction of liver oil was carried out by solvent extraction method. 50 g of ray fish liver samples were homogenized for two minutes using rotary homogenizer Bio-Gen PRO200 with 100 mL of methanol and 50 mL of chloroform. Then, another 50-mL of chloroform was added and homogenized using the homogenizer for another 30 Seconds. The mixture was diluted with 50 mL of distilled water.

Then mixtures were filtered through a Buncher funnel lined with Whatman No.42 filter paper under vacuum suction. The residue was rinsed using 20 mL of chloroform and the filter was transferred to a separator funnel. The aqueous layer was removed and the chloroform layer containing ray fish liver oil (bottom layer) was separated. It was filtered using a Buncher funnel lined with Whatman No.1 filter paper under vacuum suction. The residue was again rinsed with 20 mL of chloroform. The filtrate was passed through 3 g of anhydrous sodium sulfate to remove moisture.

Chloroform layer was transferred into a dry pre-weighed round-bottom flask. The chloroform was evaporated using a rotary vacuum evaporator at 40 °C. Extracted crude liver oil was weighed and treated with 0.02 BHT. Finally the prepared crude liver oil was stored at (-20 °C) .

Plate: 2.2 Fish oil Colour

2.2.3 Crude Oil Recovery Percentage

Below given equation was used to calculate the lipid content in the sample.

Equation: Percentage of lipid in the Ray fish liver fish

$$\% \text{ Lipid} = \frac{W_L}{Ws} \times 100$$

W_S= Weight of the sample (g)

W_L= Weight of Lipid extracted after evaporation (g)

2.2.4 Determination of fatty acid composition in Crude oil

Fatty acids profiles of selected oils were determined according to Buchgraber *et al*. (2000) method by Gas-Liquid Chromatography (GLC).

2.2.5 Preparation of free fatty acid for urea complexation

25 g of Ray fish liver oil was measured into a 250 ml per-dried conical flask. A mixture of 95% ethanol of 66 ml, distilled water of 11ml and KOH of 5.75g in pellet weight were added to the oil. The solution was refluxed at 85 ± 2 °C under nitrogen blanket for 1 hour. Then the solution was diluted with 50 ml of diluted water and mixed well. 100 mL of n-hexane was added and mixed well.

The Mixture was transferred to a separator funnel and aqueous layer containing saponified matter was separated. The n-hexane layer containing unsaponified matter was discarded. The separated aqueous layer was washed again using 100 mL of n-hexane. The n-hexane layer on top was separated by separator funnel and was discarded. The aqueous layer in bottom was acidified using 3 N HCl solution until pH reached 1. Then the solution was mixed with 50 mL of hexane and transferred to the separator funnel. The n-hexane layer containing fatty acid was separated and treated with 3 g of anhydrous sodium sulfate. Finally n-hexane was evaporated at 40 °C using a rotor evaporator. Liberated free fatty acid was weighed and stored at (-20 °C) after filling into 20 mL labeled glass vials.

2.2.6 Preparation of Omega- 3 concentrates using urea complexation

100 ml of 95% aqueous ethanol was added. The required urea amount was calculated according to the free fatty acid weight, urea to free fatty acid ratio was maintained at 6:1. The system was heated to 65°C and continuous stirring was practiced until the solution becomes crystal clear amber color solution. Then the system was rapidly transferred to a refrigerator where the temperature was adjusted to (- 9) °C and kept for 18 hours in the freezer FRIMED S.R.L AF70V/2 for crystallization.

After 18 hours, the mixture was allowed to reached room temperature 27°C. Then the fraction was separated by filtering using a Bunchner Funnel lined with Whatman No.1 filter paper. The filter is known as Non-Urea Complexing Fraction. The residue is the urea complexing fraction.

The Fraction containing Non-Urea Complexation was diluted with an equal volume of distilled water and acidified to pH 4 – 5 using freshly added 6 N HCl solutions. An equal amount of n-hexane was added and the mixture was stirred thoroughly for 1 hour using a magnetic stirrer. Then, the solution was transferred to a separator funnel to separate n-hexane layer. The n-hexane layer was treated with 3 g of anhydrous sodium sulfate to remove moisture. Vacuum facilitated filtration was practiced to remove hydrated sodium sulfate using a Buchner Funnel lined with Whatman No.1 paper. The n-hexane was evaporated using a rotary evaporated. The liberated Omega–3 fatty acids were treated with 0.02g BTH and stored in (-20°C) until analysis.

2.2.7 Preparation of fatty acid methyl ester (FAME)

Sodium methoxide (CH_3NaO) (0.5M): 0.074 g sodium methoxide was weighed accurately and dissolved in 2 mL of methanol. Internal standard (Methylheptadecanoate) (1mg/mL): 100 mg of methylheptadecanoate acid was dissolved in 100 ml of hexane.

Sample (100 mg) was weighed in to a 15 mL screw capped methylation tube and 1 mg/mL of internal standard

(Methylheptadecanoate), 2mL of 0.5M sodium methoxide and 300 UL of dichloromethane were added. Subsequently, mixture was kept in a heat block at 50°C for 30min. It was allowed to cool to room temperature and 5mL of distilled water added drop by drop and 100 UL of glacial acetic was added. Then the tube with contents will be centrifuged at 1500 rpm for 10 minutes at 5^0C and top hexane layer will be separated and added in to a 2ml GC Vial. Then against 500 UL of hexane will be added and centrifuged as mentioned above and top hexane layer will be separated and added to the same GC vial. Vials will be sealed with Para film and frozen immediately at (-20^0C) until analysis by GC.

2.2.8 Gas Liquid chromatography analysis of sample

The prepared FAME was analyzed by injecting 1 µL into GLC (Shimadzu, 14-B, Japan), equipped with a Flame Ionization Detector (FID) and a fused silica capillary column (100 m, 0.25 mm id and 0.20 µm film thickness) and attached with Chromotopac data processor (Model-CR6A, Shimadzu, Japan). Split ratio was 100:1. Injector and detector temperatures were maintained at 260˚C. Helium was used as carrier gas at flow rate of 20 mL/sec. The initial column oven temperature was maintained at 140˚C for 5 min and increased to 220˚C at the rate of 4˚C/min, then maintained at that temperature for 10 min. Fatty acids were identified by comparison of their retention time with authentic standards (SUPELCO 37 Component FAME Mix, Sigma Aldrich) (Kuksis *et al.*, 1967). The amount of each fatty acid were expressed as percentage (%) of the sum of all fatty acids in the sample.

3. Results and Discussion

3.1 Hepatosomatic Index (HSI) of Ray fish liver

The average body weights, average lengths and Liver weight of the String fish were found to be 2301.05g, 2594.17g and 2847.84g where lengths were 137.67cm, 153.49cm and 175.74cm . The liver weight were 253.69g, 289.52g and 313.98 g respectively. HSIs were calculated to be 9.05 %.Moreover the average lengths of stingray were measured to be 137.61 and 148.67 cm, 171.32 cm respectively. The HSI value of String ray fish is comparatively greater than the findings of Özyılmaz and Öksüz, 2015 which is 8.25 %. This increment may be due to the increased liver weight of String ray fish and the maturity level of fish.

Table3.1: The average length (cm), Total weight (g), Liver weight (g) and Hepatosomatic index (HSI) of the Ray fish.

Fish species	Length (cm)	Total weight (g)	Liver weight (g)	HSI (%)
Stingray-$_1$	137.67	2301.05	253.69	9.07
Stingray-$_2$	153.49	2594.17	289.52	8.96
Stingray-$_3$	175.74	2847.84	311.92	9.13

Mean±SD values were presented (n = 3)

3.2 Stingray Fish Liver Crude Oil Recovery Percentage

The average liver lipid levels of the String ray fish was 69.54 %. All of the ray fish had a large amount of lipids in their liver. However, the levels of liver lipid not varied greatly (Özyılmaz and Öksüz, 2015).

3.3 Fatty acid Profile of Ray fish crude liver oil

The predominant fatty acid in the stingray MUFA was C16 (palmitic acid), which had a value of 35.0%. Clearly, the average level of C16 in the ray fish liver oil was significantly higher than the level of the other ray fish liver oils. The total levels of PUFA in all the fish liver oils seems similar.

The total SFA percentages of String ray fish in this study were 44.2%. The total SFA percentages of ray fish liver oils from String ray that were previously reported were 34.97%, respectively (Navarro-Garcia *et al*., 2010). The percentages of total SFA in ray fish liver oil was not in agreement with the finding from the current study.

Table3.2: Fatty acid composition of stingray fish liver oil

Carbon Chain	Stingray
C12:0	4.1
C13:0	0.7
C14:00	0.3
C16:0	35.0
C17:0	0.4
C18:0	2.5
C20:0	1.2
ΣSFA	44.2
C16:1	10.2
C18:1	1.3
ΣMUFA	11.5
C18:2	0.3
C20:4	1.4
Σn6	1.7
C20:5n3	0.5
C22:6n3	0.6
Σn3	1.1
ΣPUFA	2.8
n3/n6	0.64
DHA/EPA	0.83

3.4 Fatty acid Profile of Enriched Ray fish liver oil

The predominant fatty acid in the enriched sting ray MUFA was C16 (palmitic acid), which had a value of 15.2% .Clearly, the average level of C16 in the ray fish liver oil were significantly lower than the level of the other ray fish liver oils. The total levels of PUFA in all the enriched ray fish liver oils increased significantly .

The total SFA percentages of enriched String ray fish in this study were 28.8%. The total SFA percentages of two ray fish liver oils from String ray that were previously reported was 34.97%, respectively (Navarro-Garcia *et al*., 2010). The percentages of total SFA in fish were not in agreement with the finding from the current study.

In this study, the level of ΣMUFA 11.5% in the liver oil of the stingray was lower than the levels found in string ray.(Ould El Kebir *et al*., 2005). Surprisingly, in contrast to containing high levels of C16, the liver oil of the ray fish has high amounts of EPA, and DHA, which are not generally dominant in marine fish.

In this study, the levels of DHA and EPA in ray fish liver oil differed greatly from the enriched ray fish oil. The levels of DHA and EPA in enriched ray liver oil were 9.7 % and 8.7 %, which is heigher than the levels of DHA and EPA for male *Dasyatis marmorata* that Ould El Kebir *et al*. (2007) reported.

Table 3.3: Fatty acid composition of Enriched stingray ray fish liver oil

Carbon Chain	Stingray
C12:0	1.1
C13:0	0.0
C14:00	4.0
C16:0	15.2
C17:0	0.7
C18:0	4.2
C20:0	3.6
ΣSFA	28.8
C16:1	18.5
C18:1	6.3
ΣMUFA	24.8
C18:2	1.3
C20:4	2.3
Σn6	3.6
C20:5n3	8.7
C22:6n3	9.7
Σn3	19.8
ΣPUFA	23.4
n3/n6	5.5
DHA/EPA	0.89

4. Conclusions

Ray fish by-product (liver) was obtained during dry fish processing of ray fish at Jaffna, Northern province, Sri Lanka. The liver oil was obtained through the Bligh and Dyer method gave the total recovery percentage of the ray fish liver oil was 69.54 %.. The crude enriched via urea complexation method enhance the nutritive value of ray fish liver oil by enriched ray fish liver oil with high in omega-3 content of 9.7 % and 8.7 %. Enriched ray fish liver oil from dry fish industry by-product could be suitable for applications as nutraceutical in the food industry.

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